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13. ABSTRACT (Maximum 200 Words)

A recombinant gene was constructed, encoding the soluble form of the human F1t3L gene (hFlex) at the 5′ end and the human TRAIL gene at the 3′ end. This plasmid (phFlex/TRAIL) was administrated by the hydrodynamic-based gene delivery. As a result, tumor growth was significantly delayed by hFlex/TRAIL plasmid administration. For the expression of GOD, hybrid yeast promoter-containing vector was constructed, confirming the GOD expression. In vivo studies were conducted to examine the effects of intratumoral GOD-IT administration on nude mice. As a result, tumor growth was delayed by GOD-IT administration.

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Introduction :

Prostate cancer has become the most frequently diagnosed cancer among men in the United States. Early diagnosis and new surgical, hormonal, chemical and radiotherapy regimens have contributed to improved survival and quality of life for prostate cancer patients over the past ten years (Hanks et al., 1997; Keyser et al., 1997; Kupelian et al., 1997).

Approximately 25% to 60% of patients develop elevated prostate-specific antigen levels within 5 years following treatment (Vincini et al., 1997). When prostate cancers progress to an advanced stage, they are difficult to cure. Because tumor response and treatment morbidity depend upon tumor stage, there is a need to improve treatment effectiveness for these advanced tumors while controlling morbidity. Although conventional therapies have and will continue to play major roles in the treatment of prostate cancer, greater intervention will be required to significantly enhance primary local control of prostate cancer. One such approach is through the use of gene therapy techniques to correct errant characteristics of cancerous cells, or to specifically eliminate the cells through toxic gene expression (Roth et al., 1997).

Cancer gene therapy using cytotoxic genes has attracted great attention as one of the strategies treating cancers. For successful administration of gene therapy in cancer, the therapeutic gene should be delivered specifically to tumor cells and produced gene products that act only toxic to tumor cells without killing normal cells. On the basis of this paredime, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a good candidate as a therapeutic gene due to its toxicity to tumors. TRAIL is an apoptosis-inducing member of the tumor necrosis factor (TNF) gene family (Wiley et al., 1995; Pitti et al., 1997). Recently, it has been shown that TRAIL is nontoxic systemically and that could slow the growth and in some cases, induce regression of tumor cell xenografts (Walczak et al., 1999). Preclinical studies in mice and primates have shown that administration of TRAIL can induce apoptosis in human tumors, but that no cytotoxicity to normal organ or tissue is found (Walczak et al., 1999). Obviously, differential sensitivity between normal and tumor cells to TRAIL and the mechanism of TRAIL-induced apoptosis needs to be further studied (Gura, 1997; Ashkenazi et al., 1999; Keane et al., 1999).

Previous studies demonstrate that tumors generally have lower concentrations of glucose than normal tissue. Glucose concentrations of the serum and the interstitial fluid in Walker 256 mammary carcinoma are 9.5 mM and 0.03 mM, respectively. These data suggest that the extracellular space (vascular and interstitial compartments) of solid tumors contains variable concentrations of glucose. The level of glucose probably depends on the proximity of the vascular space to the tumor cells. The clinical efficacy of TRAIL may be closely tied to the glucose concentration in the space surrounding each tumor cell. Threfore, it would be useful to develop a TRAIL-based gene therapy in conjunction with immunotherapy. Currently we are collaborating with Dr. R.V. Blackburn at ApoLife, Inc for this project. We employed a glucose oxidase (GOD) immunotoxin to further deplete glucose in the tumor. Moreover, GOD generates hydrogen peroxide. Our 1st annual report data demonstrate that TRAIL cytotoxicity is potentiated in the presence of GOD. GOD has been conjugated to a tumor specific monoclonal antibody by Dr. R.V. Blackburn. This idea is based on the observations that under physiological conditions, GOD catalyses the oxidation of β-D-glucose to D-glucose-1,5-lactone, which is subsequently hydrolysed to gluconic acid. The reaction also results in the production of hydrogen peroxide. Hydrogen peroxide exerts its effect by formation of hydroxyl radicals, which can damage various cellular components, causing DNA strand breaks, protein modification, and lipid peroxidation (Vallyathan and Shi, 1997). *In vitro* and *in vivo* studies have demonstrated that GOD has potent tumoricidal activity (Higuchi et al., 1991; Nathan and Cohn, 1981; O'Donnell-Tormey et al., 1985; Samoszuk, M.K. et al., 1989). Hydrogen peroxide produced by GOD is effective in preventing tumor growth (Higuchi et al., 1991), and its effect can be enhanced by

hydrogen peroxide decomposition inhibitors such as 3-aminotriazole, hydroxylamine and sodium azide (Higuchi et al., 1991). Moreover, by comparison to normal cells, tumor cells are more susceptible to GOD (Mavier, P. et al., 1988; Combs et al., 1993).

Body :

We had tried several times for making the replication-incompetent adenoviral vector containing the CMV promoter-drived TRAIL gene as a first step for preparation of a replication-competent adenoviral vector containing TRAIL and HSV-TK gene. However, even though successfully constructing the adenoviral shuttle vector containing TRAIL gene, this type of vector was proven to be not replicating, leading to host cell death prior to the production of progeny virus owing to the cytotoxic TRAIL expression. Therefore, we modified the vector system from adenovirus to plasmid itself. For the confirming the effect of plasmid into the mice, *in vivo* studies were performed to investigate the effects of TRAIL on tumor growth. Human prostate adenocarcinoma DU-145 tumors were established by subcutaneously injecting 2 x 10^6 cells into the dorsal surface of male athymic mice (nu/nu). The study consisted of 10 mice randomized as to control and experimental status. Tumors were measured 2-3 times per week and treatment was initiated when the tumors reached a mean volume of 100 mm^3. Mice were intravenously injected with saline or the hFlex/TRAIL recombinant plasmids (10 μg), which were kindly provided by Dr. Y. He (University of Pittsburgh). A recombinant gene was constructed, encoding the soluble form of the human Flt3L gene (hFlex) at the 5' end and the human TRAIL gene at the 3' end (Fig. 1). This design allows the TRAIL gene product to be secreted into the body circulation. An isoleucine zipper was added to the N-terminus of TRAIL. It is well known that addition of the zipper greatly enhances the trimerization of the fusion protein and dramatically increases its anti-tumor activity. The hydrodynamic-based gene delivery protocol (Liu et al., 1999) was employed to deliver the recombinant DNA plasmids every week. Although this experiment is still in progress, Figure 2 demonstrates that tumor growth is being significantly delayed by hFlex/TRAIL plasmids administration.

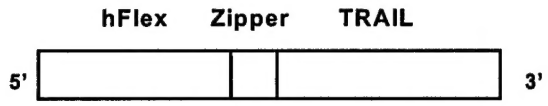


Figure 1. Schematic map of a recombinant DNA construct (phFlex/TRAIL) for TRAIL production

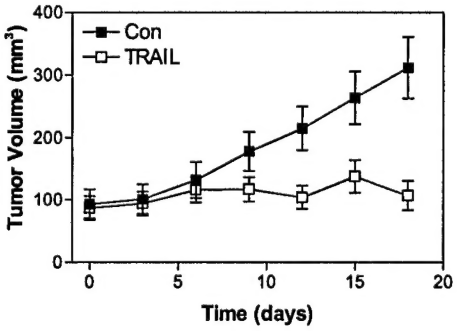


Figure 2. Effect of phFlex/TRAIL on growth of DU-145 cell xenograft tumors in nude mice.

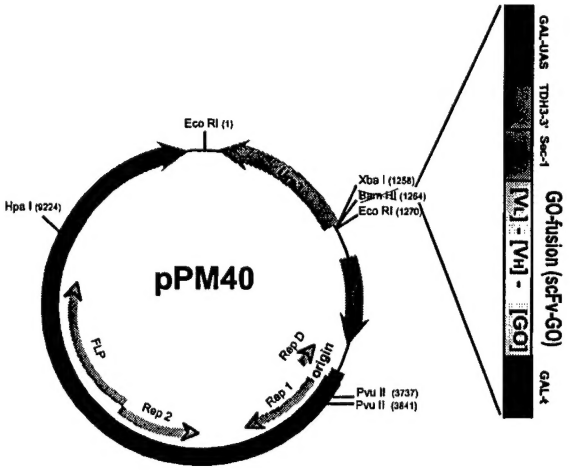


Figure 3. GOD-immunotoxin (GOD-IT) in yeast expression plasmid pPM40.

Currently we are collaborating with Dr. R.V.Blackburn at ApoLife, Inc for this project. We employed a gluocose oxidase (GOD) immunotoxin to further deplete glucose in the tumor. Moreover,

GOD generates hydrogen peroxide. Our 1st annual report data demonstrate that TRAIL cytotoxicity in potentiated in the presence of GOD. GOD has been conjugated to a tumor specific monoclonal antibody by Dr. R.V. Blackburn. This idea is based on previous observations that under physiological conditions, GOD catalyses the oxidation of β-D-glucose to D-glucose-1,5-lactone, which is subsequently hydrolysed to gluconic acid. The reaction also results in the production of hydrogen peroxide. Hydrogen peroxide exerts its effect by formation of hydroxyl radicals, which can damage various cellular components, causing DNA strand breaks, protein modification, and lipid peroxidation (Vallyathan and Shi, 1997). *In vitro* and *in vivo* studies have demonstrated that GOD has potent tumoricidal activity (Higuchi et al., 1991; Nathan and Cohn, 1981; O'Donnell-Tormey et al., 1985; Samoszuk, M.K. et al., 1989). Hydrogen peroxide produced by GOD is effective in preventing tumor growth (Higuchi et al., 1991), and its effect can be enhanced by hydrogen peroxide decomposition inhibitors such as 3-aminotriazole, hydroxylamine and sodium azide (Higuchi et al., 1991). Moreover, by comparison to normal cells, tumor cells are more susceptible to GOD (Mavier, P. et al., 1988; Combs et al., 1993).

An expression vector for a recombinant GOD immunotoxin (GOD-IT) has been constructed by Dr. R.V. Blackburn (ApoLife, Inc.) (Figure 3). This vector contains a hybrid yeast promoter that was constructed by the fusion of the upstream activating sequence (UAS) of the GAL1-10 promoter (GAL) with the downstream TDH3 promoter element (TDH3-3'). The TDH3-3' promoter segment includes the transcription initiation site, the "TATA" sequences, and the RNA polymerase binding site. This expression cassette also contains the yeast α-mating factor secretory sequences (Sec-1) and transcription termination sequences of the GAL10 gene (GAL-t). The single-chain variable region sequences (scFv) of an epithelial glycoprotein-2 (EGP-2) specific monoclonal antibody were fused to the coding region of GOD in a contiguous reading frame. The sequences encoding the 22- amino acid hydrophobic signal sequence of the native GOD were removed and the remaining DNA was fused to the scFv through a serine/glycine linker via standard recombinant techniques.

Sequences encoding the yeast α-mating signal secretory peptide were cloned into the 5' end of the fusion construct. The expression vector was transformed into yeast by electroporation, and shake flask fermentations of selected transformed clones were performed. Figure 4 shows secreted expression of crude GOD-IT (lanes #11-14) from yeast fermentation.

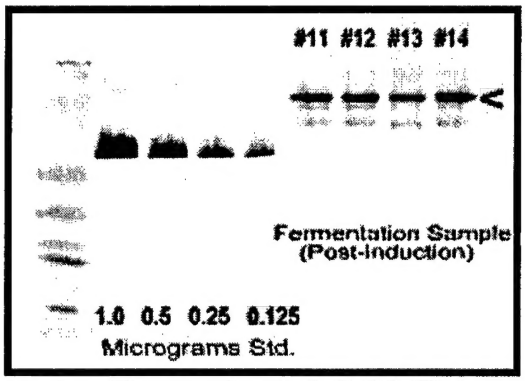
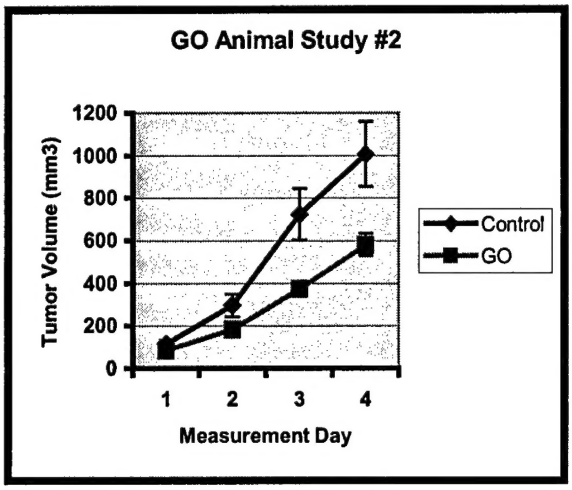


Figure 4. Expression of GOD-IT in yeast. Immunoblot of 4 time points (lane #11-14) from fermentation of a GOD-IT transformant yeast strain. Arrowhead indicates recombinant GOD-IT. Purified GOD controls (0.125-1.0 μg) were also shown.

Experiments with endoglycosidase reveal that GOD-IT indeed was glycosylated in yeast (data not shown).

In vivo studies were conducted to examine the effects of intratumoral GOD-IT administration on human colon cell tumors growing in nude mice. The study consisted of 20 mice randomized as to control and experimental status. Human colon carcinoma LS180 cells were injected into the left hind flank of nude mice. Intratumoral GOD-IT injections were begun when tumor volume reached 80-100 mm^3, at which time a single 10 μl intratumoral injection of either GOD-IT or control saline solution was given on each of 5 consecutive days. The growth of tumors was determined every third day for two weeks. Figure 5 demonstrates that tumor growth was delayed by GOD-IT administration.

Figure 5. Effect of GOD-IT on growth of LS180 cell xenograft tumors in nude mice.



Key research accomplishments

1) Glucose oxidase enhances TRAIL-induced cytotoxicity.
2) phFlex/TRAIL plasmid delayed the growth of xenograft tumors.
3) pPM40 plasmid expressing the GOD immunotoxin delayed the growth of xenograft tumors.

Reportable Outcomes

Low extracellular pH auguments TRAIL-induced apoptotic death through the mitochondria-meidated caspase signal transduction pathway. (2003) Experimental Cell Research 293: 129-143.

Conclusions

Glucose oxidase enhances TRAIL-induced cytotoxicity in vitro. And phFlex/TRAIL and pPM40 plasmid each other delayed the growth of xenograft tumors.

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